

REMARKS

Status of the Claims.

Claims 26-28, 37, and 61-63 are pending with entry of this amendment, claims 1-25, 29-36, 38-56, 57-60, being canceled and no claims being added herein. Claim 26 is amended herein for clarity. These amendments introduce no new matter. Support for the amendment to claim 26 is found page 12, lines 21-29, and page 20, lines 19-21. With respect to the "or a complement thereof" language it is noted that the specification, at page 30, lines 122-15 teaches that the target can be a chromosome (*i.e.*, double stranded DNA). One of skill in the art would readily recognize that the probe can be chosen to bind to the coding or to the antisense strand.

35 U.S.C. §112, First Paragraph - Written Description.

The Examiner rejected claims 26-28, 56, 61-63 under 35 U.S.C. §112, first paragraph, as allegedly not enabled because "the claims as written encompass a method for detecting the presence or absence of neoplastic cells . . . using probes with unknown structure and length provided said probes share a fragment with SEQ ID NO:9 and are capable of hybridizing to SEQ ID NO:9 via said common fragment under the stringent conditions recited in claims 26." The Examiner further asserted that the specification and claims lack information of the structure and function of the probes used for the claimed method and thus allegedly does not meet the written description requirement.

Applicants traverse. The Examiner is reminded that "[t]he written description requirement **does not require the applicant 'to describe exactly the subject matter claimed,** [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed. [emphasis added] " *Union Oil Co. v Atlantic Richfield et al.* 208 F.3d 989 (Fed. Cir. 2000) *citing In re Gosteli*, 872 F.2d 1008, 1012, 10 U.S.P.Q.2D (BNA) 1614, 1618 (Fed. Cir. 1989).

In the present case, claim 26 is directed to a method that comprises:

contacting a nucleic acid sample from a human patient with **a probe which specifically hybridizes under stringent conditions to a target polynucleotide sequence consisting of the sequence of SEQ ID NO:9, or the complement thereof,** wherein said stringent conditions include washing with 0.2x SSC at 65°C for 15 minutes, . . . [emphasis added]

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The probe is specifically one that “hybridizes under stringent conditions to a target polynucleotide sequence consisting of the sequence of SEQ ID NO:9 or the complement thereof”. The generation of probes specific to target sequences is routine to those of skill in the art. Indeed such probes can be generated *ad nauseum* using commercially available software tools known to those of skill in the art (*e.g.*, Oligodb available through PubMed, Sarani from Strand Genomics, Visual OMP from DNA software Ind., *etc.*). Thus, once a target nucleotide sequence is known probes that specifically hybridize to that sequence under stringent conditions are also *de facto* known to those of skill in the art.

In view of this there is simply no question that the specification, as filed, communicates to one of ordinary skill in the art that Applicants invented what is claimed. As stated by the Federal Circuit in *Union Oil*:

If lack of literal support alone were enough to support a rejection under §112, then the statement of *In re Lukach*. . . that “the invention claimed does not have to be described in *ipsis verbis* in order to satisfy the description requirement of §112, is empty verbiage.

Thus, literal language describing every claimed species is not required to meet the description requirement. To the contrary, as evidenced in *Union Oil*, guidelines and functional descriptions leading one of skill to the claimed invention are sufficient to meet the description requirements.

In the present case, the specification provides the target sequence (SEQ ID NO:9) and a desired stringency. Suitable probe sequences are readily provided by routine use of probe design software packages or even by visual inspection of the sequence (for example the complement of SEQ ID NO:9) would readily be recognized as a suitable probe.

One of ordinary skill in the art would readily appreciate that Applicants were in possession of a genus of probes that specifically hybridize under stringent conditions to a target polynucleotide sequence consisting of the sequence of SEQ ID NO:9. Consequently, given the level of skill in the art, it is readily apparent that Applicants were in possession of the claimed invention.

Accordingly the claims meet the Written Description requirement and the rejection of claims 26-28, 56, 61-63 on these grounds should be withdrawn.

35 U.S.C. §112, First Paragraph - Enablement.

Claims 26-28, 37, 56, and 61-63 were rejected under 35 U.S.C. §112, first paragraph, as allegedly not enabled because:

- 1) Applicants have allegedly failed to establish that ZABC-1 is amplified in the 20q13.2 amplicon; and
- 2) Allegedly no specific probes are recited for use in the detection of SEQ ID NO:9.

1) Amplification of ZABC-1.

Claims 26-28, 37, 56, and 61-63 were rejected under 35 U.S.C. §112, first paragraph, as allegedly not enabled. In particular, the Examiner alleged that Applicants have failed to establish that the recited sequencer (SEQ ID NO:9) is overexpressed in neoplastic cells having an amplification at 20q13.2. Applicants traverse.

Claim 26, as amended herein recites:

A method of detecting in a sample the presence or absence of **breast cancer or colorectal cancer** cells having an increased copy number of nucleic acid sequences at chromosome region 20q13.2, . . .

The prevailing scientific literature clearly establishes that ZABC1 (aka ZNF217) is amplified and overexpressed in breast cancer and colorectal cancer cells characterized by an amplification at 20Q13.

Thus, for example, Collins *et al.* (1998) *Proc. Natl. Acad. Sci., U.S.A.*, 95(15): 8703-8708), attached as Exhibit A, states:

ZNF217 is centrally located in the 260-kb common region of amplification, transcribed in multiple normal tissues, and **overexpressed in all cell lines and tumors in which it is amplified and in two in which it is not.** ZNF217 is predicted to encode alternately spliced, Kruppel-like transcription factors of 1,062 and 1,108 aa, each having a DNA-binding domain (eight C2H2 zinc fingers) and a proline-rich transcription activation domain.

* * *

ZNF217 has several features that suggest it is an oncogene involved in breast cancer. First, it is located in a narrowly defined region of recurrent maximal amplification apparently devoid of other transcribed genes.

* * *

Finally, ZNF217 was transcribed at high levels in 10 of 10 tumors and cell lines with 20q13.2 amplification and 2 without amplification (cell line 600MPE and primary tumor T4) and at low levels in 17 of 19 tumors and cell lines without amplification. Thus, although amplification appears to be the predominant mechanism leading to overexpression, transcript abundance also may be increased by other mechanisms as occur for established oncogenes such as *ERBB2*, *MYC*, and *NMYC*.

Similarly, Hidaka et al. (2000) *Clin. Cancer Res.*, 6: 2712-2717, previously submitted,

Our results suggest that **the level of 20q13.2 amplification correlates with the metastatic potential and tumor progression of CRC. The results also suggest that 20q13.2 amplification with ZNF217 is associated with increased metastatic potential.** [emphasis added] (*see abstract*)

Recently, novel zinc finger gene *ZNF217*, **which is amplified in breast cancer, was found on 20q13.2** (25). According to the recent analysis of FISH mapping of 20q in CRC metastases, the most frequent region with high-level gains was located on 20q13.1–20q13.2 (14). However, the candidate genes that are clearly associated with the metastatic potential of CRC in this region (20q13.2) remain unknown. **Our results suggest that the increased copy number of the ZNF217 ZNF217 gene may be associated with liver metastasis in CRC.** [emphasis added] (page 2715 col. 2 to page 2716, col. 1)

The scientific literature thus clearly establishes that 2013 is amplifce in breast and colorectal cancers and that ZABC1 (located within this amplication) is consequently amplified.

Applicants have clearly established that the recited sequencer (SEQ ID NO:9) is overexpressed in colorectal cancer and breast cancer cells having an amplification at 20q13.2. Accordingly the rejection on these grounds should be withdrawn.

2) ZABC-1 Specific Probes.

The rejected claims 26-28, 56, 61-63 as not enabled alleging that no probes are identified. The Examiner further alleged that using any probe, non-related nucleic acids could be detected. Applicants traverse.

Claim 26, as amended herein, recites:

contacting a nucleic acid sample from a human patient with **a probe which specifically hybridizes under stringent conditions to a target polynucleotide sequence consisting of the sequence of SEQ ID NO:9, or the complement thereof.**

The claims clearly **do not** read on the use of any probes. To the contrary, the claims, as amended, recite the use of probes that **specifically hybridize** to a nucleic acid consisting of a recited sequence (SEQ ID NO:9 or the complement there of).

The probes contemplated for use in the claimed method thus specifically bind (*e.g.* specifically detect) the recited target sequence and not other non-related sequences that may be present.

Moreover, the Examiner is reminded that "a patent need not teach **and preferably omits**, what is well-known in the art." [emphasis added] *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986) *cert. denied* 480 U.S. 947 (1987). As explained above, given a known target sequence (*e.g.* SEQ ID NO:9) the design of suitable specific probes is routine and well known to those of skill in the art.

The claimed method is thus fully enabled and the rejection of claims 26-28, 56, 61-63 on these grounds should be withdrawn.

35 U.S.C. §112, First Paragraph -- Scope.

Claims 26-28, 56, and 61-63 were rejected under 35 U.S.C. §112, first paragraph, as allegedly not enabled because:

- 1) The specification is allegedly not enabling for detecting the presence or absence of "any neoplastic cell" and;

2) The specification is alleged not enabling for a method of detecting in "any sample" the presence or absence of neoplastic cells having an increased copy number.

Applicants traverse.

1) "Any neoplastic cell".

The Examiner rejected the claims as not enabled because the specification is allegedly not enabling for detecting the presence or absence of "any neoplastic cell". Claim 26, as amended herein recites:

26. A method of detecting in a sample the presence or absence of **breast cancer or colorectal cancer cells having an increased copy number of nucleic acid sequences at chromosome region 20q13.2**, the method comprising

The claim simply is not directed to detecting any neoplastic cell. To the contrary, as the highlighted language reveals, the claim is directed to a method of detecting breast cancer or colorectal cancer cell having an increased copy number of nucleic acid sequences at chromosome region 20q13.2. The references discussed above clearly establish that breast and colorectal cancers have an amplification at 20Q13 and that ZABC1 is amplified therein.

The scope of the claims thus fully supported. Accordingly, Applicants have met the requirements of 35 U.S.C. §112, first paragraph, and the rejection of claims 28, 56, and 61-63 should be withdrawn.

2) "In any sample".

Claims 26-28, 37, 56, and 61-63 were rejected under 35 U.S.C. §112, first paragraph, as allegedly overbroad because the specification is allegedly not enabling for a method "for detecting in 'any sample' the presence or absence of neoplastic cells having an increased number of nucleic acid sequences at chromosome region 20q13.2. In particular, the Examiner alleges that only breast cancer has been shown to have an increased number of copies of genes detected by the 1.5 M probe, the RMC20C001 probe and that it is unpredictable that any cancer cell or any cancer tissue would have an increased copy number of SEQ ID NO:9. . . ". As explained above, claim 26 expressly recites:

26. A method of detecting in a sample the presence or absence of **breast cancer or colorectal cancer cells having an increased copy number of nucleic acid sequences at chromosome region 20q13.2,**

In view of the highlighted language, the claim is plainly directed to detecting the presence or absence of breast cancer or colorectal cancer cells having increased copy number of nucleic acid sequences at chromosome region 20q13.2.

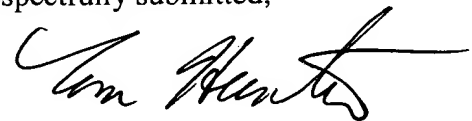
Applicants have clearly established that 20Q13 amplicon occurs in breast cancer and in colorectal cancer cells and that ZABC1 is amplified therein. Accordingly, the pending claim is fully enabled and the rejection on these grounds should be withdrawn.

In view of the foregoing, Applicants believes all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested. **Should the Examiner seek to maintain the rejections, Applicants hereby expressly request, on the record, that the Examiner call Applicants to arrange a telephone interview with the Examiner and the Examiner's supervisor.**

If a telephone conference would expedite prosecution of this application, the Examiner is invited to telephone the undersigned at (510) 769-3513.

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Positional cloning of *ZNF217* and *NABC1*: Genes amplified at 20q13.2 and overexpressed in breast carcinoma

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ABSTRACT We report here the molecular cloning of an ≈ 1 -Mb region of recurrent amplification at 20q13.2 in breast cancer and other tumors and the delineation of a 260-kb common region of amplification. Analysis of the 1-Mb region produced evidence for five genes, *ZNF217*, *ZNF218*, and *NABC1*, *PIC1L* (*PIC1*-like), *CYP24*, and a pseudogene *CRP* (Cyclophilin Related Pseudogene). *ZNF217* and *NABC1* emerged as strong candidate oncogenes and were characterized in detail. *NABC1* is predicted to encode a 585-aa protein of unknown function and is overexpressed in most but not all breast cancer cell lines in which it was amplified. *ZNF217* is centrally located in the 260-kb common region of amplification, transcribed in multiple normal tissues, and overexpressed in all cell lines and tumors in which it is amplified and in two in which it is not. *ZNF217* is predicted to encode alternately spliced, Kruppel-like transcription factors of 1,062 and 1,108 aa, each having a DNA-binding domain (eight C2H2 zinc fingers) and a proline-rich transcription activation domain.

Studies in which comparative genomic hybridization was used have revealed ≈ 20 regions of recurrent increased DNA sequence copy number in breast tumors (1–3). These regions are predicted to encode dominantly acting genes that may play a role in tumor progression or response to therapy. To date, three of these regions have been associated with established oncogenes: *ERBB2* at 17q12, *MYC* at 8q24, and *CCND1* and *EMS1* at 11q13. In breast cancer, *ERBB2* and *CCND1/EMS1* amplification and overexpression are associated with decreased life expectancy (4, 5), whereas *MYC* amplification has been associated with lymph node involvement, advanced stage, and an increased rate of relapse (6, 7). Efforts are now underway in several laboratories to identify oncogenes in the other regions of amplification.

Amplification at 20q13 is particularly interesting because this aberration occurs in a variety of tumor types and is associated with aggressive tumor behavior. The initial comparative genomic hybridization study showed increased copy number involving 20q13 in 40% of breast cancer cell lines and 18% of primary breast tumors (2). Since then, other comparative genomic hybridization studies have revealed copy number gains at 20q13 in greater than 25% of cancers of the ovary (8), colon (9), head and neck (10), brain (11), and

pancreas (12). This region was analyzed at higher resolution in breast tumors and cell lines by using fluorescence *in situ* hybridization (FISH), and a 1.5-Mb-wide region of recurrent amplification containing the cosmid clone RMC20C001 was defined (13, 14). Interphase FISH with RMC20C001 revealed low level (>1.5 -fold) and high level (>3 -fold) amplification in 29% and 7% of breast cancers, respectively (15). High level amplification was associated with an aggressive tumor phenotype (15, 16).

Increased copy number of chromosome 20q in cultured cells also has been associated with phenotypes characteristic of progressing tumors, including immortalization and genomic instability. Specifically, increased copy number at 20q11-qter has been observed frequently in human uroepithelial cells (17) and keratinocytes (18) after transfection with HPV16 E7 or HPV16, respectively. In addition, increased copy number at 20q13.2 has been associated with p53-independent genomic instability in some HPV16 E7-transfected human uroepithelial cell lines (19).

These studies suggest that increased expression of one or more genes on 20q and especially at 20q13.2 contribute to the evolution of breast and other solid tumors. Several candidate genes, including *AIB1* (20), *BTAK* (21), *CAS* (22), and *TFAP2C* (23), that may contribute to the various phenotypes associated with increased copy number involving 20q have been identified. However, we describe here a region of recurrent increased copy number at 20q13.2 that does not involve these genes. Extensive analysis of this region identified four previously unknown genes, one known gene, and a pseudogene. Two of the novel genes, designated *ZNF217* and *NABC1* (Novel Amplified in Breast Cancer-1), were amplified and overexpressed in multiple cell lines and tumors and were characterized extensively.

MATERIALS AND METHODS

Cell and Tissue Sources. Karyotypically normal, finite lifespan human mammary epithelial cells 161RM, 48RM, and

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: FISH, fluorescence *in situ* hybridization; STS, sequence-tagged site; QPCR, quantitative PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EST, expressed sequence tag; BCM, Baylor College of Medicine.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF041259 and AF041260).

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184RM and the immortalized line 184B5 were obtained from M. Stampfer (Lawrence Berkeley National Laboratory) and cultured as described (24). Cell lines SKBR3, BT474, MDA-MB-436, HBL100, MDA-MB-435, HS758T, and MCF7 were obtained from American Type Culture Collection (ATCC) and cultured as suggested by ATCC. MDA10 was obtained from the National Cancer Institute. The cell line 600MPE was provided by H. Smith (California Pacific Medical Center, San Francisco) and cultured as described (25). Primary breast tumor samples were obtained from the San Francisco Bay Area Breast Cancer SPORE repository or from Tampere University.

Physical Map. YAC clones from the Center d'Etudes du Polymorphisme Humain and Integrated Molecular Analysis of Gene Expression Consortium cDNAs were identified in the Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genomic Research web site (<http://www.genome.wi.mit.edu>) and obtained from Research Genetics (Huntsville, AL). P1 and Bacterial Artificial Chromosome libraries were screened by using PCR with sequence tagged sites (STSs) from 20q13.2 as described (13) or as recommended by the supplier (Research Genetics or Genome Systems, St. Louis). All clones were mapped to normal metaphase cells by using FISH to confirm cytogenetic location. The position and physical relationship of each clone to its neighbors were determined and verified by many methods, including STS content mapping, Southern blot hybridization, fiber FISH, DNA fingerprinting, and genome sequence analysis.

Copy Number Analysis with Interphase FISH. All samples were screened for amplification at 20q13.2 by using FISH with probes prepared from clones RMC20C001, RMC20B4095, and/or a pool of clones made up of RMC20P4030, RMC20P4040, RMC20P4038, RMC20P4039, RMC20P4041, and RMC20P4042. Samples found to be amplified by using one of these probes were analyzed more extensively by using probes prepared from clones listed in Table 1. Dual-color FISH was performed as reported (14) by using a test probe and a reference probe at 20p prepared from RMC20B235 containing D20S894. Fifty to 100 intact, nonoverlapping nuclei were scored for each probe. Nuclei with more than 20 signals were scored as >20 because of the difficulty of accurate signal enumeration at high copy number. Tumor-infiltrating leukocytes were excluded based on their small size. FISH to normal fibroblasts was performed to confirm that the hybridization efficiency for each probe was high.

Gene Fragment Identification. A Lambda ZAP II (Stratagene) cDNA library constructed from the adenocarcinoma cell line Caco-2 (ATCC HTB 37) was screened with probes identified during direct cDNA selection and/or exon trapping as described (26). Selection of cDNAs by using the Genetrapp system was performed as recommended by the supplier (Life Technologies, Grand Island, NY). Exon trapping was accomplished as described (27). Direct selection of cDNA clones from a library generated from poly(A)⁺ RNA isolated from the cell line BT474 (ATCC HTB 20) was performed as described (28). Gene fragments selected by these methods were sequenced (SEQwright, Houston) and mapped back onto the clones from which they were selected by using PCR and/or Southern hybridization.

Northern Blot Analysis. Total RNA from tumors or cell lines was extracted by Trizol separation as recommended by the supplier (Life Technologies). Each RNA sample (15 μ g) was heated at 60°C for 15 min and loaded onto a 0.67 M formaldehyde/1.0 \times Mops/1.0% (wt/vol) agarose denaturing gel. After electrophoresis, RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) with 20 \times SSC and immobilized by UV crosslinking and baking at 70°C for 2 h. Hybridizations were carried out in Church buffer (29). All membranes were washed in 0.2 \times SSC/0.1% SDS at 60°C before detection. Hybridized probe was detected autoradio-

Table 1. Overlapping genomic clones across a \sim 1-Mb region at 20q13.2

Clone identification	Type	Size, kb	Library coordinate
RMC20B4166	BAC	130	22614
RMC20B4123	BAC	94	189C22
RMC20P4067	P1	74	91B2
RMC20P4068	P1	50	100D12
RMC20P4042	P1	57	103D9
RMC20P4041	P1	56	86C1
RMC20B4095	BAC	80	140H15
RMC20P4038	P1	63	112G8
RMC20P4039	P1	74	34A6
RMC20P4040	P1	80	24H1
RMC20P4030	P1	102	124G6
RMC20B4097	BAC	160	133E8
RMC20P4007	P1	56	31D11
RMC20B4127	BAC	120	27P15
RMC20B4103	BAC	77	188A15
RMC20B4130	BAC	137	341H15
RMC20P4185	P1	83	1141D7
RMC20B4188	BAC	103	163G21
RMC20B4189	BAC	81	165E16
RMC20P4002	P1	83	12C6
RMC20B4109	BAC	103	62A6
RMC20B4108	BAC	48	432B9
RMC20P4028	P1	68	118G11
RMC20P4010	P1	75	36F10
RMC20B4099	BAC	160	146L11
RMC20P4018	P1	64	77A10
RMC20P4069	P1	77	412B5
RMC20B4122	BAC	108	39M7
RMC20B4174	BAC	101	278I13
RMC20B4087	BAC	87	96J14

Clones are listed in approximate genomic order from centromere to telomere. These clones are available from the Lawrence Berkeley National Laboratory/University of California, San Francisco Resource for Molecular Cytogenetics (RMC) upon request via (<http://rmc-www.lbl.gov/>).

graphically or by using a Molecular Dynamics Phosphor-Imager.

Quantitative PCR (QPCR). Total RNA was isolated from cell lines and frozen tumor tissue by using Trizol separation. Frozen tissue was disrupted in Trizol by using a hand homogenizer before separation. RNA pellets were dissolved in deionized formamide to a concentration >1 μ g/ μ l and stored at -20°C. Total RNA was treated with DNase I (Life Technologies) to remove any contaminating genomic DNA before reverse transcription. This RNA (15 ng) was reverse transcribed in a 100- μ l reaction consisting of 1 \times PCR buffer II (Perkin-Elmer; 50 mM KCl/10 mM Tris, pH 8.3), 5.5 mM MgCl₂, 500 μ M each 2'-deoxynucleoside 5'-triphosphate, 2.5 μ M random hexamers, 0.4 unit/ μ l RNase inhibitor, and 2.5 unit/ μ l Superscript II reverse transcriptase (Life Technologies). Reverse transcription was carried out at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min and then incubated at 4°C. As a control for genomic contamination, 15 ng of DNase-treated RNA was processed as described above except that reverse transcriptase was not added.

The relative abundances of *ZNF217* and *GAPDH* mRNAs were assessed by using QPCR (30). A 105-bp fragment in exon 3 of *ZNF217* was amplified by using primers: F1, 5'-GATGTTACTCCTCCTCCGGATG, and R1, 5'-CACACTTGGCCTGTATCTGCA. A TaqMan probe, 5'-FAM (6-carboxy fluorescein)-AAAGAGAAGCAAACGGAGACCGCAGC-3'-TAMRA (6-carboxy-tetramethylrhodamine), was included during QPCR. PCR primers and the TaqMan probe for *GAPDH* were obtained from Perkin-Elmer. QPCR ampli-

cation was carried out in triplicate in 50- μ l reaction volumes consisting of 1 \times PCR buffer A (Perkin-Elmer), 5.5 mM MgCl₂, 0.4 μ M each primer, 200 μ M each 2'-deoxynucleoside 5'-triphosphate, 100 nM probe, and 0.025 unit/ μ l Taq Gold (Perkin-Elmer). cDNA (500 pg) or no reverse transcriptase control RNA was amplified according to the thermal profile: 95°C for 10 min (95°C for 15 s, 60°C for 1 min) \times 40 cycles and incubated at 25°C.

The number of cycles needed for FAM fluorescence from the *ZNF217* and *GAPDH* reactions to cross a predetermined threshold was measured for each sample during QPCR by using an Applied Biosystems 7700 (31). The amount of starting *ZNF217* mRNA relative to that for *GAPDH* for each sample, i , was calculated as $2^{\Delta\Delta N_i}$, where ΔN_i was the difference in the number of cycles needed for the FAM fluorescence intensity for the *ZNF217* and *GAPDH* reactions to reach a threshold value. All measurements were normalized to the *ZNF217*/*GAPDH* ratio for the cell line MDA10, which was analyzed in every experiment. Thus, the *ZNF217*/*GAPDH* relative expression level for each sample was $RE_i = 2^{(\Delta N_i - \Delta N_{cal})}$, where ΔN_{cal} was the difference in the number of cycles needed for the FAM fluorescence intensities from the *ZNF217* and *GAPDH* reactions to reach a threshold value in the MDA10 reference.

Expression of Exons, cDNAs, and ESTs (Expressed Sequence Tags). The presence of specific exons, cDNAs or ESTs in first-strand cDNA from the cell line BT474 or multiple cDNA libraries (Life Technologies) was determined by PCR amplification. Sequence-specific primers were designed by using MacVector software after screening for repetitive elements by using the Baylor College of Medicine (BCM) web server. Amplification reactions in 10- μ l volumes were carried out with 1 μ l of first-strand cDNA or 50 ng of library cDNA template by using capillary thermocyclers (Idaho Technologies, Idaho Falls, ID). The first-strand cDNA from BT474 was prepared from 2 μ g of total RNA that had been digested with RNase-free DNase I. The RNA was incubated with 250 ng of oligo(dT)₁₂₋₁₈ and 25 ng of random hexamers at 70°C for 10 min and then placed on ice. First-strand synthesis was carried out in 1 \times first-strand buffer, 0.5 mM each dATP, dCTP, dGTP, and dTTP, and 5 mM DTT (Life Technologies). After 1 h, the reaction was stopped by incubation at 55°C for 10 min. The remaining RNA was degraded by addition of RNase H, and the mixture was put at 55°C for an additional 10 min.

DNA Sequencing. SEQwright performed all cDNA sequencing. Sequence manipulation and analysis was performed by using MACVECTOR (Oxford Molecular, Campbell, CA) and the BCM nucleic acid and protein search launchers (<http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html>). All genome sequencing was performed in the Lawrence Berkeley National Laboratory Human Genome Center by using a directed sequencing strategy (32).

DNA and Protein Sequence Analysis. DNA sequences and predicted proteins were searched against public nucleic acid and protein databases by using BLAST, BLASTX, TBLASTX, BLASTX+BEAUTY, BLASTP, and BLASTP + BEAUTY algorithms (33) via the BCM search launcher and MACVECTOR software. Repetitive elements were identified by using the BCM REPEATMASKER. Genomic sequence was analyzed by using XGRAIL (34), GRAIL1a, SORFIND (35), and GENOTATOR (36). Protein sequence was analyzed by using BLOCKS and PPSEARCH via BCM and MacVector software.

RESULTS

Molecular cloning of a 1-Mb Region at 20q13.2. Overlapping P1 and BAC clones were assembled across a \approx 1-Mb interval of 20q13.2 containing RMC20C001 to provide the framework for high-resolution analysis of DNA sequence copy number and for gene discovery. Thirty overlapping BAC and P1 clones and connecting STSs and ESTs are shown in Fig. 1a. The clone

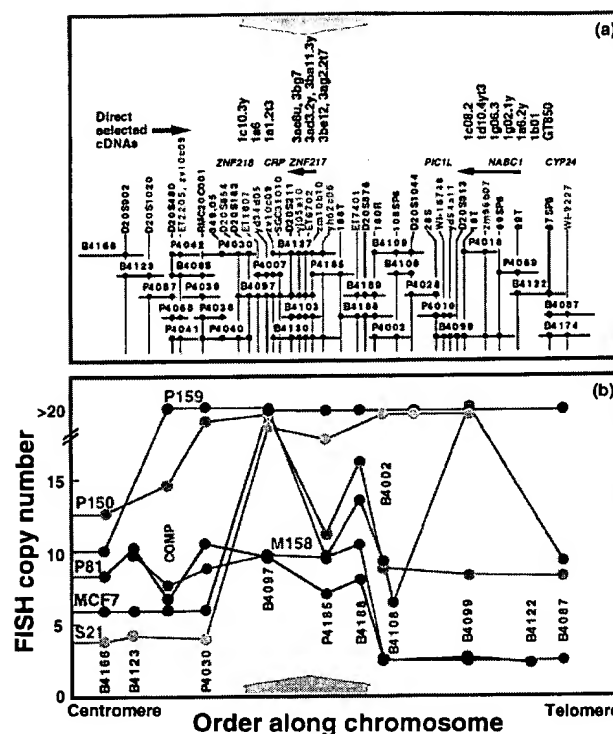


FIG. 1. (a) Physical map of 20q13.2 between D20S902 and WI-9227. Clones that make up the map are indicated as horizontal lines. The lengths of the lines are proportional to the sizes of the clones. The last five digits of the RMC identifiers listed in Table 1 identify clones. Mapped STSs and ESTs in this region are shown as vertical black and red lines, respectively. ●, Clones that tested positive for STS or EST content by PCR. The approximate genome locations of five genes and a pseudogene are shown above the map. Arrowheads indicate transcriptional polarity where known. The approximate locations of 17 of 30 directly selected cDNA fragments are shown at the top. (b) FISH analysis of DNA sequence copy number in five informative breast tumors and one breast cancer cell line. Probes used in this study are indicated. The probe labeled COMP comprised clones P4030, P4040, P4038, P4039, P4041, and P4042. The vertical axis shows the number of hybridization signals produced by FISH with each probe. The number of signals is truncated at 20 because hybridization signal enumeration was difficult above this level. The x axis scales are the same for a and b. The results of six FISH studies are color coded by sample. Solid lines connect the measurements made for each sample. A \approx 260-kb region of common maximal amplification is indicated by gray arrows above (a) and below (b).

names, library coordinates, and sizes are listed in Table 1. The region extends from D20S902 to WI-9227 and is covered with Center d'Etudes du Polymorphisme Humain YACs 847g7, 845f3, and 820f5. A comprehensive description of the map and its genome sequence will be published elsewhere (C.C., unpublished data).

DNA Sequence Copy Number Analysis. Approximately 280 breast cancer tissue samples and cell lines were screened for increased DNA sequence copy number by using interphase FISH. Seventeen primary breast tumors and cell lines with $>3\times$ amplification relative to 20p were identified and analyzed for copy number at several points across the 1-Mb region by using FISH. In 11 of these, most of the 1-Mb region was amplified. However, the pattern of amplification in six suggested a \approx 260-kb-wide region of maximal amplification extending from RMC20B4097 to RMC20B4188 (Fig. 1b). Copy number in the cell line MCF-7 and tumor S21 increased sharply at RMC20B4097 and extended distally, whereas in tumors P81 and M158, copy number increased sharply at RMC20B4188 and extended proximally. The copy number in tumors P150

and P159 also was high in the common region of maximal amplification. However, tumor P159 (Fig. 1b) and several others (not shown) had complex amplification patterns that hinted at the existence of other sites of amplification.

Gene Discovery. Clones composing the ≈ 1 -Mb contig were analyzed for gene content by using genomic sequencing (37), exon trapping (38), and direct cDNA selection (28) with cDNA prepared from the breast cancer cell line BT474. The region defined by RMC20P4185, RMC20B4188, and RMC20P4002 was analyzed twice by using exon trapping and cDNA selection because of the paucity of putative gene fragments that were recovered. Thirty-one trapped exons and 30 cDNAs were identified and sequenced. Whereas trapped exons were distributed broadly over the 1-Mb region, the cDNAs clustered as illustrated in Fig. 1a. ESTs either were identified by searching dBEST with sequence from genomic clones, trapped exons, and cDNA clones or were identified in the Whitehead and National Center for Biotechnology Information databases.

Several methods were used to determine the transcription patterns of the trapped exons, cDNA fragments, and ESTs. First-strand cDNA synthesized from total RNA isolated from the breast cancer cell line BT474 and DNA from multiple cDNA libraries were screened by using PCR with primer pairs designed from the DNA fragments. In addition, Northern blots of multiple tissue poly(A)⁺ mRNA samples and breast cancer cell line total RNA were probed. Independent DNA fragments that had the same approximate genome locations and patterns of transcription were assumed to be segments of one gene. These investigations provided evidence for three novel transcribed genes designated *ZNF217*, *ZNF218*, and *NABC1*. These were considered to be oncogene candidates and were characterized further (see below).

Evidence also was found for two other genes and a pseudogene in the 1-Mb region. These included *CRP* (Cyclophilin Related Pseudogene), *CYP24* (vitamin D 24-hydroxylase), and *PIC1L* (*PIC1*-like). *CRP* was discovered in the genomic sequence by its significant DNA and amino acid alignment to cyclophilin (39). However, its ORF was disrupted by in-frame stop codons. *CYP24* was previously reported to be involved in the regulation of calcium homeostasis (40). *PIC1L* was identified based on the homology of its predicted protein sequence to that for *PIC1* (93% similarity), a nuclear body protein that interacts with *PML* in promyelocytic leukemia (41). Reverse transcription-PCR analyses of total mRNA from several breast cancer cell lines including BT474 revealed no evidence of expression for any of these sequences, and they were not analyzed further.

Transcribed Gene Descriptions. *ZNF218* was inferred from the transcription patterns detected by ET2205, direct selected cDNAs 1c10.3y, 1a6, and 1a1.2t3, and EST zv10c09. Hybridization of each of these DNA fragments to multitissue Northern blots revealed transcripts of ≈ 11 , 8, and 7 kb in several tissues, including prostate, testis, ovary, and intestine, and a single transcript of 7 kb in the liver (data not shown). However, they did not detect transcription in breast cancer cell lines amplified at RMC20B4097. Interestingly, EST zv10c09 partially overlapped ET2205 and also mapped to a second site ≈ 200 kb distal to ET2205, suggesting that it is composed of two exons separated by a large intron.

NABC1 was identified by seven direct selected cDNA fragments (1c08.2, 1d10.4yt3, 1g06.3, 1g02.1y, 1a6.2y, 1b01, and GT850; Fig. 1a). Hybridization of each these DNA fragments to multitissue Northern blots revealed a ≈ 3 -kb transcript present at high abundance in brain and prostate and at lower abundance in testis, intestine, and colon (data not shown). Hybridization to Northern blots of total RNA from breast cancer cell lines and cultured epithelial cells derived from reduction mammosplastics showed the 3-kb transcript to be present at high levels in three of four cell lines amplified at RMC20B4099 but not detectable in normal epithelial cells

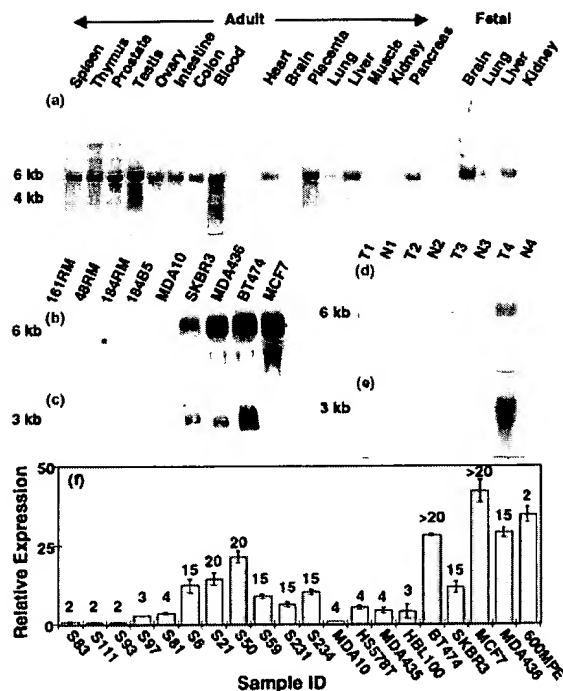


FIG. 2. Expression of *ZNF217* and *NABC1*. (a) Hybridization of a *ZNF217* probe to multiple tissue poly(A)⁺ Northern blots. (b) Northern blot analysis of *ZNF217* transcription in three independent karyotypically normal breast epithelial cultures of finite lifespan (161RM, 48RM, 185RM), one immortalized line (184BS) and five breast cancer cell lines (MDA10, SKBR3, MDA436, BT474, MCF7). The breast cancer cell lines carry 4, 10–15, 10–15, >20, and >20 copies of the region encoding *ZNF217*, respectively. (c) Northern blot analysis of *NABC1* transcription in the same cell lines. (d) Analysis of *ZNF217* transcription in four paired breast tumor/normal epithelium samples. Lanes containing RNA from normal and tumor tissues are labeled N and T, respectively. (e) Analysis of *NABC1* transcription by using the same membrane described in d. (f) QPCR analysis of *ZNF217* mRNA abundance relative to that for *GAPDH* in 11 primary breast tumors and nine breast cancer cell lines. DNA sequence copy number at the *ZNF217* locus determined by FISH with RMC20B4097 is indicated above each QPCR measurement.

(Fig. 2c). Northern hybridization to total RNA extracted from four primary breast tumors and the corresponding normal tissue revealed high level transcription in at least one tumor (Fig. 2e). However, *NABC1* was not in the common region of maximal amplification, and transcription was not detected in the breast cancer cell line MCF7 in which it was amplified (Fig. 2c). A 2813-bp *NABC1* cDNA was isolated from a colon carcinoma cDNA library and sequenced (accession no. AF041260). Analysis of the cDNA sequence identified a 1752-bp ORF predicted to encode a protein of 584 amino acids with no significant homology to other proteins (Fig. 3a).

ZNF217 was inferred from the pattern of transcription detected by exon ET6702, ESTs yj05a10 and zm10b10, and direct selected cDNAs 3ae8u, 3bg7, 3ad3.2y, 3ba11.3y, 3be12, and 3ag2.2t7. These DNA fragments mapped to the distal end of RMC20B4097 (Fig. 1a). They detected a major ≈ 6 -kb transcript in most tissues except adult brain and fetal kidney. An additional ≈ 4 -kb transcript was present in testis (Fig. 2a). The relative abundance of the major *ZNF217* transcript was higher in breast cancer cell lines that were amplified at RMC204097 (MCF7, BT474, SKBR3, and MDA436) than in a nonamplified breast cancer cell line (MDA10) or cultured epithelial cells derived from reduction mammosplastics (161RM, 48RM, 184RM, and 184B5; Fig. 2b). *ZNF217* was transcribed at high level in one of four primary tumors analyzed by Northern blot hybridization (Fig. 2d). *ZNF217*

(a)	
MGNQMSVPQVRVEDQENPEAEYTDNASALNGVPPVSTHTVQHLEEDVLGIVSKTDNV	59
ATSSPETTEISAVADANGKNGLEAKPEAPAAKSRFFMLSLRPVPGRTGDQADSSLG	118
VKLDVSSNKAPANKDPSWTLTPVAAGPGQDTDKTPGHAPAQDKVLSAARDPTLLPET	177
GGAGGEAPSKPKDSSFFDKPFKLDKGQEVPGDSQQAERAEHQDKVDEVPGLSGQSD	236
VPAKDIVDGKEKEGELGTADCSVPDGPGLTAKDDSQAAIAENNNISIMSFKTLV	295
SPNKAETKDPEDTGAESPTTSADLXSKANFTSQBTQAGKNSKGCNPSGHTQSVTT	354
PEPAKEGTKEKSQPTSLPLGKLFWKKSVDKSVPTGAEENVVCSPEVETIKSKEVESAL	413
QTVLDNEGDAAPETPAKLRKEESKPRSLMAFLRQMSVKDGGGITHSEINGKDSQ	472
TSDESTKITTPPEPTGAPQKQEGSSKDKSAEMNKQKSNQKAEKPAQCTEQATV	531
DTNSLQNGDKLQKRPKRQSLGGFFGLGPKRMLDAQVQTDVPSIGPVGKPK*	583
(b)	
MQSKVTGNMPTQSLMLMYMDGPEVIGSSLSGSPMEMEDALSMKGTAVVPFRATQENKVIQI	59
EGYMLPDCNFCSTQTFTHSEDLNKHVLMQMRPTLCEPAVLRAEAYLSPLDKSQVRTEPP	118
KEKNCKENEFSCVCGQTFRVAFDVSECHHRTKDSFTYGCNMGCRFFKEPFLKDHMT	177
HNKSGARSKLQGLSSPATINEVQVHAESISSPYKICNVCGLFFPKKESLIEHAK	236
VHTKKTAFGTSSAQTDSPQGGMPSSREDPLQLFNLRPKSHPETGKKPVRCIPQLDPFTT	295
PQAWLATKGVKIAICQEVKESQEGSTDDNDSSSEKELGETNKGSCAGLSQEKKECKHS	354
HGEAPSVDAADPKLPSSKEKPTHCSECKAFRTYHQLVLSRVHKKDRRAGAESPTMSVD	413
GRQPGTCSPLAALPDENGAVDRGEGGSEDSGLPEGIHLKNDGGKIKHLTSRE	472
CSYCGKFFRSNTYLLHILRTHTEGKPKYCFYAAQKTSRLYHLERHHEKQTDVAA	531
EVKNDGNQDTEADLTAADSAQTKNLKRFPDGAQKDTGSPPAKQLKEMPSVFNVLGSA	590
VLSPAKHDTQDFHKNAAADSDAKVNKNPTPAYLDLLKRSVAVETQANNLICRTKADVTP	649
PPDGTHTNLVSPKEKQTEATAADCRYPVSVDCEHEKPLNLVSGALHNCPSISLSKLIP	708
SITCPFFCTFKTYFEVLVLMHQLRLEHYKYNPDVHKCNKRKSLRSRRGCPALLGKDVPP	767
LSSFCPKPKSAFPAQSKSLPSAKGQSPGPGKAPLTSGIDSSLTAPSNLKSHPQQN	826
VGVQGAATRQQCSEMPKTSVSPAPDKTKRPETKLKPLVAPSQPTLGSSNINGSIDYV	885
AKNDSPPWAPGRDYFCNRSASNTAAEFGEPLPKRLKSVVVALVDVQPGANYRRGYDLPK	944
YHVRGITSLLPQDCVYPSQALPPKPRFLSSSEVDSPNLTVQKPYGSGPLYTCVPAG	1003
SPASSSTLEGKRPVSYQHLNSMAQRNYENFIGNAHYRPNDKKTGLGGCQCLLPMLKN	1062
FTSFEKRMVKATEISCDCTVHKTYEESARNTTV*	1096

FIG. 3. Predicted amino acid sequences. (a) *NABC1*. (b) *ZNF217*. Eight putative C2H2 zinc finger domains are indicated in bold. A repeated motif in exon 1 is double underlined. Lines above the text indicate the positions of two putative tyrosine phosphorylation sites predicted by the PROSITE pattern search algorithm. A putative proline-rich transcription activation domain is encoded by amino acids 757–1005 and is composed of 16% proline. Exon boundaries were determined from the cDNA and genomic sequences and are marked with arrows. Exon 1 codes for 455 aa and exon 3 codes for 517 aa. The amino acid sequence of the alternately processed exon 4 is underlined.

expression was studied further by using the QPCR. Eleven primary tumors and nine breast cancer cell lines were analyzed. Six of the tumors and four of the cell lines carried >15 copies of the region defined by RMC20B4097. *ZNF217* was transcribed at high level in all of these (Fig. 2f). Significantly, *ZNF217* also was transcribed at high level in the breast cancer cell line 600MPE in which it was not amplified.

***ZNF217* cDNA and Genomic Organization.** The genome and cDNA organization for *ZNF217* shown in Fig. 4 were deter-

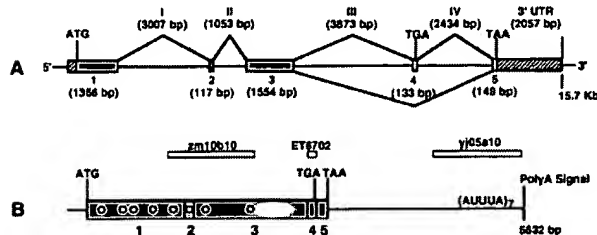


FIG. 4. Genomic organization of *ZNF217*. (A) The genomic organization of the five exons with encoded initiation and termination codons that make up *ZNF217*. Exon 4 encodes a TGA termination codon and is alternatively processed. Hatched boxes represent known 5'- and 3'-untranslated regions (UTR) in the cDNA. The sizes of exons and introns appear below and above the map, respectively. (B) The map of the 5632-bp *ZNF217* cDNA. Vertical bars represent exon boundaries. The relative positions of the predicted eight C2H2 Kruppel-like zinc finger motifs are indicated by white circles. The position of the proline-rich putative transcription activator domain is shown as a hatched oval. AUUUA motifs are indicated in the 3'-untranslated region. The relative locations of three ESTs are shown in boxes.

mined by sequencing directly selected cDNAs, trapped exons, ESTs, genome DNA, and cDNAs isolated from cDNA libraries. These analyses determined the complete 5,632-bp *ZNF217* cDNA sequence (accession no. AF041259) and the 15.7-kb genome sequence in which it is encoded. Alignment of the cDNA and genome sequences revealed five coding exons flanked by canonical splice sites (Table 2). Analysis of the aligned consensus cDNA sequences identified alternately spliced ORFs of 3,186 and 3,324 bp with the structures indicated in Fig. 4. Alternative processing of the 133-bp exon 4 was suggested by the alignment of cDNAs from colon carcinoma and HeLa cell lines and exon ET6702.

Computational analysis of the translated ORFs indicated that *ZNF217* encodes proteins of 1,062 and 1,108 aa (Fig. 3b). Both predicted proteins contained eight C2H2 zinc finger DNA-binding motifs. In addition, a proline-rich (16–20%) domain was located at residues 757–1,005 (Fig. 3b). Proline-rich domains have been shown to function as transcriptional activators in genes such as *CTF/NF-1* (42). An imperfect polyadenylation signal (AATATA) was found 14 bases upstream of the poly(A) tail of a cDNA clone defined by EST yj05a10. Analysis of the *ZNF217* 3'-untranslated region revealed seven AUUUA motifs but no repetitive or conserved elements. The region of the mRNA sequence from nucleotide 5,211 to nucleotide 5,567 was composed of 44% uridine and contained five of the seven AUUUA motifs.

DISCUSSION

Several studies have suggested that amplification of genes on 20q contribute to neoplastic transformation and/or progression in breast cancer and other solid tumors. These include the association of increased copy number at 20q13 with high tumor grade and an aggressive tumor phenotype (14–16, 43) and the association between increased copy number at 20q and immortalization and genome instability in HPV-16-transfected cells (17–19). Several candidate oncogenes have been reported as amplified on 20q, but none of these are in the 1-Mb region of amplification analyzed in this study. *AIB1* (20) and *CAS* (22) map proximal to this 1-Mb region at 20q12, and 20q13.1, respectively. *TFAP2C* (23) maps distal to the region by dual-color FISH (data not shown), and *BTAK* (21) maps distal to the 1-Mb region in Center d'Etudes du Polymorphisme Humain YAC 757e8.

Four previously unknown genes, *PIC1L*, *ZNF217*, *ZNF218*, and *NABC1*, and the known gene *CYP24* were located in the 1-Mb region analyzed in this study. Three of these, *CYP24*, *ZNF218*, and *PIC1L* were excluded as candidate breast cancer genes because they were not expressed in breast cancer cell lines amplified at 20q13.2. *NABC1* was highly expressed in three amplified breast cancer cell lines (SKBR3, BT474, and MDA436) and in one tumor without amplification at RMC20B4099. However, it was not expressed in MCF7 in which it is highly amplified (Fig. 1b). Furthermore, it mapped outside the 260-kb region of common maximal amplification defined in this study. Nevertheless, it remains a tantalizing candidate and merits further characterization.

ZNF217 has several features that suggest it is an oncogene involved in breast cancer. First, it is located in a narrowly defined region of recurrent maximal amplification apparently devoid of other transcribed genes. Moreover, DNA sequence analysis suggests that *ZNF217* encodes a transcription factor with 8 C2H2 Kruppel-like (44) DNA-binding motifs and a proline-rich transcription activator domain. Kruppel-like transcription factors have been implicated in several human malignancies, including *EV11*, *PLZF*, and *PLM* in leukemia, *BCL6* in lymphomas, *GLI1* in glioblastoma, *WT1* in Wilms tumor, and *ZBP89* in gastric carcinoma. The presence of mRNA-destabilizing AU-rich elements in its 3'-untranslated region further suggests that *ZNF217* is an oncogene because these

Table 2. Sequence of exon boundaries in *ZNF217*

No.	Exon, bp	Intron, bp	Exon-intron boundaries	
			Splice acceptor junction	Splice donor junction
1	1,366*			ATCCATCTGG/ g taagctgccct
2	117	3,007	ttttccttta ag /ATAAAAATGA	ACGCATACAG/ g taagaacttt
3	1,554	1,053	ttcttgcc ttag /GTGAAAAACC	ACGTTAGAAG/ g tattgcatgag
4	133	3,873	tttttcaat cag /GAAAAAGGCC	GGGAAAAAG/ g tgagcatatgt
5	149*	2,434	tttttcc ttag /GTCTTGGTGG	

Exon sequences are shown in upper case; intron sequences are shown in lower case.

*Translated portions.

motifs have been implicated in the destabilization of mRNA transcripts of protooncogenes and nuclear transcription factors (e.g., *FOS*, *JUN*, *MYC*) and cytokines (*GM-CSF*, *IL-3*, *b-IFN*) (45). Finally, *ZNF217* was transcribed at high levels in 10 of 10 tumors and cell lines with 20q13.2 amplification and 2 without amplification (cell line 600MPE and primary tumor T4) and at low levels in 17 of 19 tumors and cell lines without amplification. Thus, although amplification appears to be the predominant mechanism leading to overexpression, transcript abundance also may be increased by other mechanisms as occur for established oncogenes such as *ERBB2*, *MYC*, and *NMYC*.

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